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Ras Activity in the *Drosophila* Prothoracic Gland Regulates Body Size and Developmental Rate via Ecdysone Release

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Summary

Background: In *Drosophila*, each of the three larval instars ends with a molt, triggered by release of steroid molting hormone ecdysone from the prothoracic gland (PG). Because all growth occurs during the larval stages, final body size depends on both the larval growth rate and the duration of each larval stage, which in turn might be regulated by the timing of ecdysone release.
Results: Here, we show that the expression of activated *Ras*, *PI3 kinase* (*PI3K*), or *Raf* specifically in the PG reduces body size, whereas activated *Ras* or *PI3K*, but not *Raf*, increases PG cell size. In contrast, expression of either dominant-negative (dn) *Ras*, *Raf*, or *PI3K* increases body size and prolongs the larval stages, leading to delayed pupariation, whereas expression of dn-*PI3K,* but not of dn-*Raf* or dn-*Ras*, reduces PG cell size. To test the possibility that altered ecdysone release is responsible for these phenotypes, we measured larval ecdysone levels indirectly, via the transcriptional activation of two ecdysone targets, E74A and E74B. We found that the activation of Ras within the PG induces precocious ecdysone release, whereas expression of either dn-*PI3K* or dn-*Raf* in the PG greatly attenuates the [ecdysone] increase that causes growth cessation and pupariation onset.
Conclusions: We conclude that Ras activity in the PG regulates body size and the duration of each larval stage by regulating ecdysone release. We also suggest that ecdysone release is regulated in two ways: a PI3K-dependent growth-promoting effect on PG cells, and a Raf-dependent step that may involve the transcriptional regulation of ecdysone biosynthetic genes.

Introduction

The final body size of an organism reflects both its growth rate and the duration of its growth phase. The growth rate of an organism is regulated in large part by nutrient availability, to which the organism responds with both cell-autonomous and -nonautonomous mechanisms. At the cellular level, growth requires the activation of the insulin receptor (InR) pathway, which includes the downstream enzymes PI3 kinase (PI3K), Akt, and Target of Rapamycin: In both mice and flies, activation of this pathway either within particular tissues or in the entire organism promotes autonomous growth [1–5]. Cell-nonautonomous growth regulation occurs in many cases via the release of insulin-related growth factors. For example, in *Drosophila*, ablation of several neurosecretory cells expressing insulin-like peptides causes a growth defect [6], and mice lacking an anterior pituitary are dwarves because they fail to release growth hormone [7], an important activator of insulin-like growth-factor release [8]. In addition, flies mutant for the amino acid transporter encoded by *slimfast* [9] also show cell-nonautonomous defects in growth rate.

Final body size is also regulated by the duration of the growth phase. In insects, because all growth occurs during the larval stages, adult size will be determined by larval size at the time of the pupal molt. The timing of this molt, in turn, is regulated by the release of the steroid molting hormone ecdysone by cells of the prothoracic gland (PG). In the tobacco hornworm, *Manduca sexta*, in which this regulation is best understood, ecdysone release is triggered by the release of prothoracicotropic hormone (PTTH) from neurosecretory cells within the larval brain [10]. The PTTH release that triggers metamorphosis requires a drop in the juvenile hormone (JH) titer, a drop that occurs when larvae reach the critical weight [11, 12]. Molting in *Drosophila* might be regulated by similar mechanisms. The duration of the larval phase can be artificially shortened or lengthened by triggering premature or delayed ecdysone release, respectively. Premature ecdysone release leads to the rapid development of small adults, whereas delayed ecdysone release leads to the delayed appearance of large adults [11, 12]. Thus, final body size in insects is regulated at least in part by the interactions of JH, PTTH, and ecdysone, which together control the duration of the growth phase.

In *Manduca*, PTTH application not only increases [cyclic AMP] ([cAMP]) and [Ca^{2+}] within the PG, a process that is both necessary and sufficient for ecdysone release [13, 14], but it also activates Erk, presumably via the Raf pathway, and this activation has uncertain physiological significance [15]. This mechanism is species specific because *Drosophila* PG cells, unlike *Manduca* PG cells, do not release ecdysone in response to increased [cAMP] [16].

Here, we use the *Drosophila Gal4/UAS* system to modulate Ras signaling in PG cells. We find that expression of constitutively active *Ras* shortens larval stages, leading to precocious pupariation, and that expression of activated *Ras* or its downstream effectors *Raf* and *PI3K* confers reduced pupal and adult size. Furthermore, expression of activated *Ras* and *PI3K*, but not of activated *Raf*, increases PG cell size. In contrast, we find that expression of dominant-negative (dn) *Ras*, *Raf*, or *PI3K* in PG cells prolongs the larval stages, leading to delayed pupariation, and confers increased pupal and adult size. Furthermore, expression of dn-*PI3K*, but not of dn-*Ras* or dn-*Raf*, reduces PG cell size. To test the possibility that these size and developmental-rate effects result from altered ecdysone release, we used quantitative RT-PCR to measure the transcriptional activation of the ecdysone signaling targets *E74A* and *E74B* [17] in larvae with altered Ras signaling. We find that expression of activated Ras in PG cells causes

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precocious release of ecdysone beginning at about 72 hr after egg laying (AEL); we suggest that this precocious release promotes premature pupariation and the rapid appearance of small pupae and adults. In contrast, we find that inhibiting Raf or PI3K in the PG strongly attenuates the rate at which ecdysone levels increase during the third-instar stage, an attenuation that we suggest inhibits the onset of pupariation, causing prolonged larval growth and the delayed appearance of large pupae and adults. We conclude that ecdysone release regulates both body size and developmental rate and that this release is regulated by two mechanisms: a PI3K-dependent activation of PG cell growth, and a separate Raf-dependent mechanism.

Results

Expression Pattern of amn^{c651} and amn^{X8}

The *Drosophila amnesiac (amn)* gene, which is thought to encode three neuropeptides [18], is essential for a number of physiological processes, including learning and memory, normal ethanol sensitivity, and proper growth control within peripheral nerves [18–20]. Two independent insertions of *P(Gal4)* elements into *amn* have been reported: amn^{c651} [21] and amn^{X8} [19], which is an *amn* deletion caused by an imprecise excision of the *P(Gal4)* insertion amn^{28a} and which retains full Gal4 activity (V. Hall and M.S., unpublished data).

The *Gal4* expression pattern of amn^{c651} within adult heads has been reported [21]. To visualize the cells expressing Gal4 within developing larvae, we crossed amn^{c651} and amn^{X8} flies to flies carrying a nuclear-localized GFP expressed under *UAS* control, *UAS-GFP(nls)* [22]. We found that amn^{c651} first produces detectable Gal4 activity in the ring gland of first-instar larvae (Figure 1A). In third-instar larvae, Gal4 activity is detected in cells of the prothoracic gland (PG) of the ring gland (Figures 1A and 1B), as well as in five cells within each brain lobe and about two cells per hemisegment in the ventral ganglion (Figure 1B). The amn^{X8} *Gal4* element is also expressed strongly in the PG, but it is expressed more widely, both outside of the central nervous system (CNS, Figure 1A) and within the CNS (Figure 1B), than the amn^{c651} *Gal4* element. The nonspecific, non-nuclear-localized fluorescence within the gut is present in wild-type larvae (Figure 1A), which lack GFP, and thus represents GFP-independent background fluorescence. However, we are able to visualize specific, nuclear-localized GFP over this background. For example, higher-magnification images (Figure 1A, far right panels) show specific, nuclear-localized GFP within a subset of cells of the gut from amn^{X8}, but not amn^{c651} or wild-type larvae. amn^{X8} is expressed in a few other tissues in addition to CNS and gut (for example, fat body and salivary gland, not shown). In contrast, we are unable to detect amn^{c651} expression outside of the nervous system (Figure 1A and not shown). This extremely restricted expression pattern is similar to what was observed for amn^{c651} expression in the adult [21].

Regulation of Body Size by the Ras Pathway

We used amn^{c651} and amn^{X8} to test the effects of altering the activity of Ras or downstream effectors within the PG. We found that adults bearing amn^{c651} and either of two transgenes expressing the constitutively active Ras^{V12} under *UAS* control, denoted *UAS-*Ras^{V12}*(II)* and *UAS-*Ras^{V12}*(III)* [23, 24], are morphologically normal but significantly reduced in size (Figure 2 and Table 1). This phenotype is not simply a consequence of Ras overexpression because flies carrying amn^{c651} and *UAS-*Ras^{+} exhibit normal body size (Figure 2 and Table 1). To determine whether this size phenotype was a result of reduced cell size or cell number, we measured total wing hair-cell number and wing hair-cell density and found that the reduced wing area was accompanied by a reduction in both cell number and cell size. A similar property was previously observed in other fly mutants exhibiting reduced size [25, 3]. Although flies bearing amn^{c651} and *UAS-*Ras^{V12}*(II)* are significantly reduced in size (Figure 2 and Table 1), the presence of escapers of normal size prevents the growth reduction by Ras^{V12} *(II)* from reaching the same extent as the reduction conferred by Ras^{V12} *(III)*. Thus, expression of either activated *Ras* transgene within the PG reduces fly size.

We found that flies bearing amn^{X8} and *UAS-*Ras^{V12}*(II)* or *UAS-*Ras^{V12} *(III)* produce very small but morphologically normal third-instar larvae and pupae (Figure 2 and Table 1), which fail to eclose. In contrast, flies bearing amn^{X8} and *UAS-*Ras^{+} are normal in size and viability. Thus, both *Gal4* insertions into *amn* confer small body size in the presence of *UAS-*Ras^{V12}.

Ras^{V12} exerts its effects through several targets, including Raf, PI3 kinase (PI3K), and Ral-GDS [26–28]. To identify the effector(s) responsible for the small-size phenotype, we introduced the gain-of-function transgenes Ral^{V20}, Raf^{F20}, and *PI3K-CAAX* [29–31] under *UAS* control into flies bearing amn^{c651}. We found that expression of Ral^{V20} had no effect on body size. In contrast, expression of either Raf^{F20} or *PI3K-CAAX* reduced body size to about half of the extent of Ras^{V12} (Figure 2 and Table 1). These results suggest that Ras^{V12} exerts its effects via both Raf and PI3K. *PI3K-CAAX* or Raf^{F20} expressed under amn^{X8} control conferred embryonic lethality.

The experiments described above demonstrate that activated Ras, PI3K, and Raf within the PG are sufficient to reduce fly size. To determine whether these activities are necessary for size regulation, we introduced the dominant-negative Ras^{N17}, $PI3K^{D954A}$, and Raf^{K497M} [24, 31, 32] alleles under *UAS* control into flies bearing amn^{c651}. Expression of any of these three constructs increased pupal and adult size (Figure 2 and Table 1). The increased size of the wing epidermal cells in flies expressing $PI3K^{D954A}$ and Raf^{K497M} constructs suggests that most, if not all, of this increased size results from increased cell size rather than cell number (Table 1). We found a similar increase in pupal length when Ras^{N17}, $PI3K^{D954A}$, and Raf^{K497M} expression was driven by amn^{X8} (Figure 2 and Table 1). These results demonstrate that a reduction in Ras signaling in the PG increases fly size.

Effects of Altered Ras Signaling on Rate of Growth and Development

The large flies produced by amn^{X8}- or amn^{c651}-driven Ras^{N17}, $PI3K^{D954A}$, and Raf^{K497M} develop (proceed

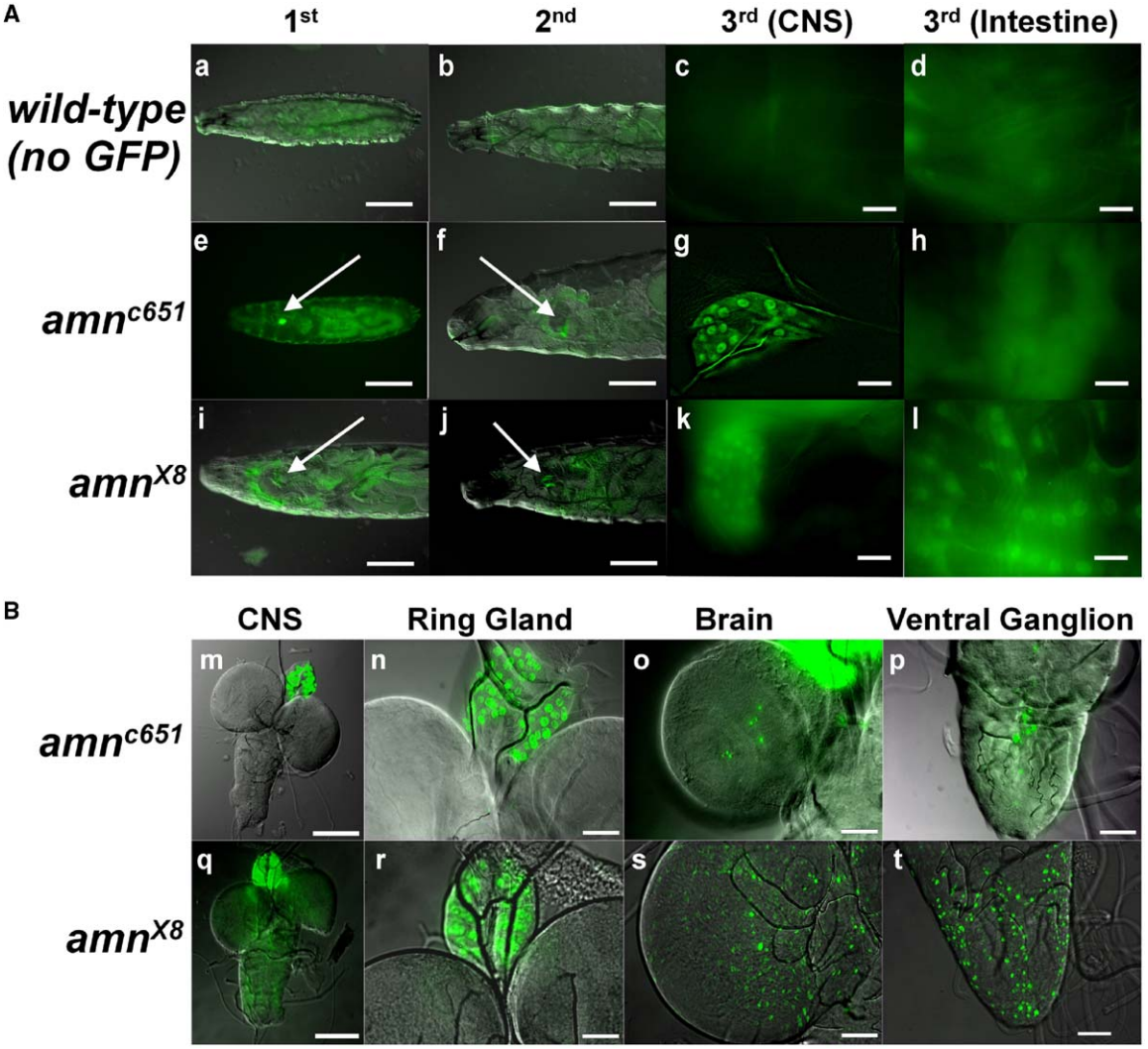

Figure 1. Temporal and Spatial Expression Patterns of amn^{c651} and amn^{X8} Visualized via Nuclear-Localized GFP

(A) Expression patterns of wild-type, amn^{c651}, and amn^{X8} larvae during the first-, second-, and third-instar stages. Larvae from wild-type flies do not carry GFP, and thus background fluorescence in the intestinal lumen is GFP independent. Both amn^{c651} and amn^{X8} are expressed in the ring gland during each larval stage (shown by arrows in (A_e, A_f, A_i, and A_j); however, amn^{X8} is expressed in many other cell types. For example, the far-right panels show a magnified image of the gut in which specific, nuclear-localized GFP expression can clearly be seen over background fluorescence in amn^{X8} larvae but not in wild-type or amn^{c651} larvae. Measure bars are 200 μm for (A_a, A_b, A_e, A_f, A_i, and A_j) and 50 μm for (A_c, A_d, A_g, A_h, A_k, and A_l). The fluorescent nuclei in (A_k) appear blurry because they were photographed through the cuticle.

(B) Expression patterns of amn^{c651} and amn^{X8} in the central nervous system in wandering third-instar larvae. (A_m–$_p$) amn^{c651} is expressed in prothoracic gland cells of the ring gland and is expressed in very few cells within the brain and ventral ganglion. (A_q–$_t$) amn^{X8} is expressed strongly in prothoracic gland cells of the ring gland and is expressed in many more cells of the brain and ventral ganglion than amn^{c651}. Measure bars are 50 μm for (A_m, A_o, A_p, A_r, A_s, and A_t) and 200 μm for (A_m and A_q).

through larval stages) more slowly than normal, consistent with their increased size. In particular, we found that in developing larvae staged at 12 hr intervals, flies heterozygous for amn^{X8} or amn^{c651} and either *UAS-PI3K*^{D954A} or *UAS-Raf*^{K497M} pupariate about 36 hr after wild-type controls (Figures 3A and 3B), which results mostly from a prolonged third instar stage. Larvae in which Ras^{N17} expression is driven by amn^{c651} also undergo delayed pupariation (about 48 hr after wild-type controls, Figure 3A). However, most of this developmental delay is the result of a prolonged second-instar stage. It is unclear why Ras^{N17} appears to act earlier than *PI3K*^{D954A} or *Raf*^{K497M}. It is possible that the Ras^{N17} transgene is expressed at a higher level relative to endogenous *Ras* than are the *PI3K*^{D954A} or *Raf*^{K497M} transgenes relative to endogenous *PI3K* and *Raf*, respectively.

In contrast, larvae heterozygous for amn^{X8} and *UAS-Ras*^{V12} develop more rapidly than normal and begin pupariation about 18 hr before wild-type larvae or controls in which amn^{X8} drives *UAS-Ras*$^{+}$ (Figure 3B). In addition, amn^{c651}-driven *Ras*^{V12} larvae begin wandering and pupariate about 24 hr before controls (data not shown). However, the time to pupariation of the amn^{c651}-driven

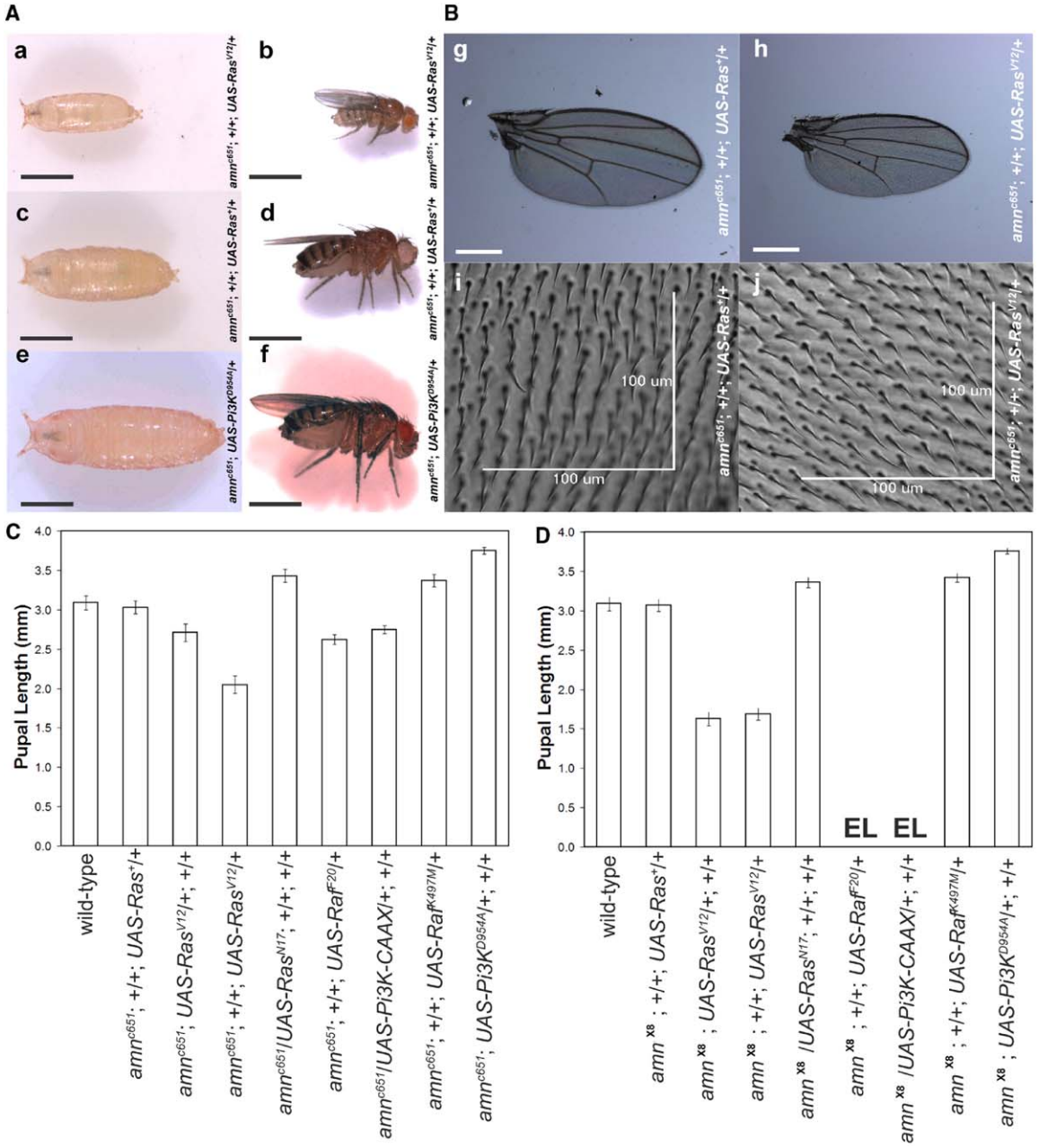

Figure 2. Altered Ras Signaling under the Control of *amn*^{c651} and *amn*^{X8} Affects Fly Size

(A) Photographs of pupal and adult size. (A_a and A_b) Expression of *Ras*^{V12} under *amn*^{c651} control decreases pupal and adult size. (A_c and A_d) Expression of *Ras*$^{+}$ under *amn*^{c651} control produces pupae and adults of normal size. (A_e and A_f) Expression of *Pi3K*^{D954A} under *amn*^{c651} control increases pupal and adult size. All measure bars are 1 mm.

(B) Photographs of wing size and wing hair-cell density. (B_g and B_i), Expression of *Ras*$^{+}$ under *amn*^{c651} control produces wings of normal size with normal wing hair-cell density. (B_h) and (B_j), expression of *Ras*^{V12} under *amn*^{c651} control decreases wing size but increases wing hair-cell density. Measure bars for (B_g) and (B_h) are 200 μm.

(C and D) Graphs depicting the effects of Ras signaling under *amn*^{c651} and *amn*^{X8} control on female pupal size. Wild-type flies were *w*^{1118} and isogenic for the second and third chromosome. Analysis is described in the text. The number of pupae or adults tested is ≥10. Mean ± standard error of the mean (SEM) is shown. All relevant p values are ≤0.01. EL represents embryonic lethality.

Ras^{V12} larvae is much more variable than in wild-type controls: The mean time to pupariation has a standard deviation of less than 12 hr for control larvae but greater than 18 hr for *amn*^{c651}-driven *Ras*^{V12} larvae. These results, taken together, indicate that the altered size conferred by alterations in Ras signaling might occur at least in part by alteration in the duration of the larval phases (rate of development) and, hence, the duration of the growth phase.

The growth rate (rate of size increase) is also affected in larvae expressing *Ras*^{V12} in the PG. For example, until about 84 hr AEL, larvae in which *Ras*^{V12} is driven by

Table 1. Altered Ras Signaling under the Control of *amn*^{c651} and *amn*^{X8} Quantitatively Affects Fly Size

	Pupal Length (mm)		Adult Length (mm)				Wing Hair-Cell Number/100 μm^2	Average Hair Cells/Wing
	Female	Male	Female	Male	Average Weight (mg)	Wing Area (mm^2)		
Wild-Type	3.09 ± 0.09	2.95 ± 0.11	2.62 ± 0.09	2.51 ± 0.10	0.84	1.72	121	2150
amn^{c651}; +/+; *UAS-Ras*$^{+}$/+	3.03 ± 0.08	2.94 ± 0.07	2.66 ± 0.07	2.53 ± 0.07	0.97	1.77	124	2200
amn^{c651}; *UAS-Ras*^{V12}/+; +/+	2.71 ± 0.11	2.74 ± 0.12	2.54 ± 0.10	2.67 ± 0.10	0.64	1.47	134	1970
amn^{c651}; +/+; *UAS-Ras*^{V12}/+	2.05 ± 0.11	1.98 ± 0.08	1.98 ± 0.06	1.99 ± 0.07	0.41	1.08	144	1560
amn^{c651}/*UAS-Ras*^{N17}; +/+; +/+	3.43 ± 0.08	N.A.	2.89 ± 0.09	N.A.	1.21	2.23	100	2300
amn^{c651}; +/+; *UAS-Raf*^{F20}/+	2.62 ± 0.06	2.55 ± 0.05	2.46 ± 0.04	PL	0.62	1.35	139	1875
amn^{c651}/*UAS-PI3K-CAAX*; +/+; +/+	2.75 ± 0.05	N.A.	2.46 ± 0.06	N.A.	0.75	1.38	138	1900
amn^{c651}; +/+; *UAS-Raf*^{K497M}/+	3.37 ± 0.08	3.52 ± 0.06	2.85 ± 0.04	2.59 ± 0.04	1.19	2.16	104	2250
amn^{c651}; *UAS-PI3K*^{D954A}/+; +/+	3.75 ± 0.04	3.91 ± 0.07	3.03 ± 0.04	2.77 ± 0.04	1.31	2.25	100	2250
Wild-Type	3.09 ± 0.09	2.95 ± 0.11	2.62 ± 0.09	2.51 ± 0.10	0.84	1.72	121	2150
amn^{X8}; +/+; *UAS-Ras*$^{+}$/+	3.07 ± 0.08	2.92 ± 0.10	2.64 ± 0.08	2.56 ± 0.08	0.91	1.77	119	2100
amn^{X8}; *UAS-Ras*^{V12}/+; +/+	1.63 ± 0.09	1.62 ± 0.12	PL	PL	PL	PL	PL	PL
amn^{X8}; +/+; *UAS-Ras*^{V12}/+	1.69 ± 0.08	1.64 ± 0.08	PL	PL	PL	PL	PL	PL
amn^{X8}/*UAS-Ras*^{N17}; +/+; +/+	3.36 ± 0.07	N.A.	2.99 ± 0.06	N.A.	1.18	1.8	115	2300
amn^{X8}; +/+; *UAS-Raf*^{F20}/+	EL	EL	EL	EL	EL	EL	EL	EL
amn^{X8}/*UAS-PI3K-CAAX*; +/+; +/+	EL	EL	EL	EL	EL	EL	EL	EL
amn^{X8}; +/+; *UAS-Raf*^{K497M}/+	3.42 ± 0.06	3.10 ± 0.08	2.68 ± 0.03	2.45 ± 0.04	1.22	1.98	119	2350
amn^{X8}; *UAS-PI3K*^{D954A}/+; +/+	3.76 ± 0.04	3.76 ± 0.04	PL	PL	PL	PL	PL	PL

Wild-type flies were w^{1118} and isogenic for the second and third chromosome. PL denotes pupal lethal, and EL denotes embryonic lethal. Mean ± SEM is indicated, and all relevant p values $\leq$ 0.01. For pupal and adult lengths, n $\geq$ 10; for average adult weight, n $\geq$ 50; for wing area, n = 1; and for wing hair-cell number/100 μm^2, n = 3.

amn^{c651} are similar in size to wild-type or larvae in which *Ras*$^{+}$ is driven by *amn*^{c651} (Figure 3C). At this time, about one-third of the *Ras*^{V12}-expressing larvae appear to stop growing (Figure 3C). These larvae begin to pupariate but fail to develop and ultimately die. The other two-thirds of these larvae grow, although more slowly than wild-type larvae, and can form the small pupae reported in Figure 2 and Table 1. All such pupae that we have observed are able to form viable adults. These results suggest that the expression of activated Ras in the PG might reduce the growth rate and thus that the reduced final body size in these larvae might reflect both a reduced growth rate and an increased rate of development.

Effects of Altered Ras Signaling on PG Cell Size

Because activated Ras can cause increased growth and proliferation, we were interested in determining whether altering Ras activity within the PG would affect PG cell size or cell number. We found that driving either *Ras*^{V12} or *PI3K-CAAX* with *amn*^{c651} increased PG cell size, with no effect on cell number, whereas driving *PI3K*^{D954A} with *amn*^{c651} reduced PG cell size (Figure 4). Expression of *Ras*^{N17}, *Raf*^{F20}, or *Raf*^{K497M} had no effect on PG cell size (Figure 4). We conclude that Ras^{V12} regulates fly size and developmental rate by two mechanisms: a PI3K-dependent effect on PG size, and a Raf-dependent step that does not involve PG growth. The observation that Ras^{N17}, which inhibits Ras^{+} activity by preventing Ras activation [33], fails to reduce PG cell size is consistent with the possibility that although Ras^{V12} is sufficient to activate PI3K, endogenous Ras activity in the PG is not necessary for PI3K activity under normal physiological conditions, presumably because PI3K can be activated in Ras-independent ways. A similar finding was reported previously in studies of the *Drosophila* wing [5]. However, an alternative possibility is that *amn*^{c651} fails to drive *Ras*^{N17} levels sufficiently to inhibit Ras^{+} completely.

Effects of Ras Signaling on Ecdysone Release

PG cells release the molting hormone ecdysone in response to PTTH release from neurosecretory cells within the brain [10]. The *Ras*^{V12}- or *PI3K-CAAX*-induced PG hypertrophy and the *PI3K*^{D954A}-induced hypotrophy raised the possibility that altered fly size and duration of the larval phases resulted from altered ecdysone release. In this view, enhanced or precocious ecdysone release would cause premature reduction of growth rate and premature pupariation and, hence, the formation of small pupae and adults. In contrast, delayed or reduced ecdysone release would delay pupariation and thus prolong the growth phase and enable the delayed formation of large pupae and adults. To test this possibility, we collected larvae with altered Ras signaling, staged developmentally at 12 hr intervals, and measured ecdysone activity in these larvae as a function of both genotype and stage of development. To measure ecdysone activity, we used an indirect reporter system: We prepared RNA from the staged larvae and used quantitative RT-PCR to measure transcriptional activation of the ecdysone signaling targets E74A and E74B [17], as well as an internal control gene,

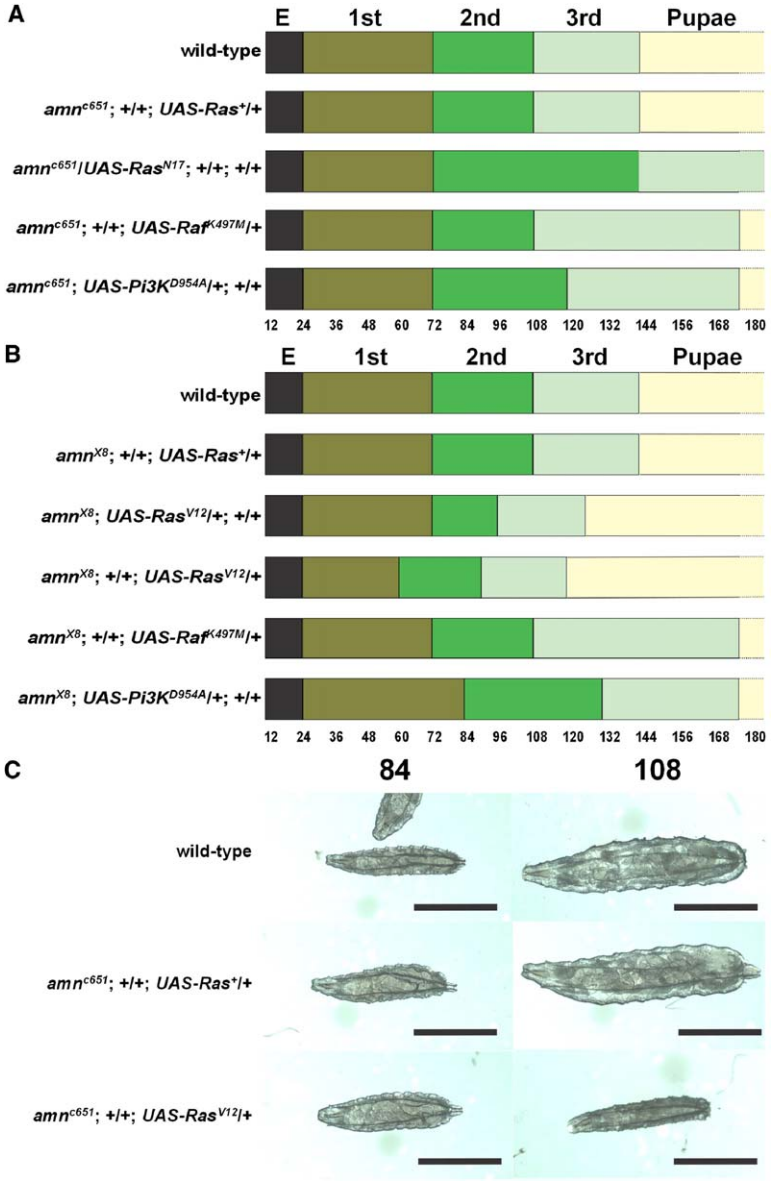

Figure 3. Ras Signaling under the Control of amn^{c651} and amn^{X8} Affects the Rate of Larval Growth and Development

(A) Expression of the indicated transgenes under amn^{c651} control.

(B) Expression of the indicated transgenes under amn^{X8} control. Wild-type flies were w^{1118} and isogenic for the second and third chromosome. Larvae were staged at 12 hr intervals after egg laying (AEL) as described in the text. The developmental stages of an approximately equal number of males and females were determined by morphology of mouth hooks and anterior spiracles. Vertical bars indicate that 50% of flies have transitioned to the next stage. Larvae were generated from ten mating pairs of the appropriate cross.

(C) Comparison of the length of wild-type larvae, or larvae that express Ras^{+} or Ras^{V12} under the control of amn^{c651} at the indicated time points (AEL). Wild-type larvae were w^{1118} and isogenic for the second and third chromosomes. Measure bar is 1 mm.

RpL13A. E74A and E74B were chosen because both genes are direct targets of ecdysone signaling but respond differentially to ecdysone concentrations. In particular, E74B is abundant at intermediate ecdysone levels but inhibited at high ecdysone concentrations, whereas E74A transcripts are present only at high ecdysone concentrations [34]. These properties predict that a rise in E74B levels will precede a rise in E74A levels, when ecdysone titers rise during the third larval instar and metamorphosis [34].

We found that in wild-type control larvae (either amn^{c651}or amn^{X8} crossed to *UAS-Ras*$^{+}$ (Figure 5) or flies bearing each *UAS-transgene* crossed to mothers lacking *Gal4* [not shown]), E74B levels began increasing early in the third-instar stage (at about 96 hr AEL), peaked at 120 hr AEL, and then dropped to low levels at 144 hr AEL (Figure 5A), at which time pupariation begins. E74A levels began to rise at 132 hr AEL and were maintained at high levels for at least the next 36 hr (Figure 5B).

In contrast, in larvae in which Ras signaling in the PG was inhibited through amn^{c651}- or amn^{X8}-driven expression of either $Pi3K^{D954A}$ or Raf^{K497M}, ecdysone levels rose abnormally slowly during the third-instar period. In particular, E74B levels were significantly lower in these larvae than in wild-type larvae from 84 to 132 hr AEL (Figure 5A). The appearance of the E74A spike was also delayed about 36 hr, which corresponds to the 36 hr delay in the onset of pupariation (Figure 5B). We suggest that this abnormally slow increase in ecdysone release is responsible for prolonging the third-instar stage, which in turn enables excessive growth to occur and, hence, the delayed appearance of large adults.

In contrast, expression of activated Ras in the PG conferred a precocious rise in ecdysone activity. In par-

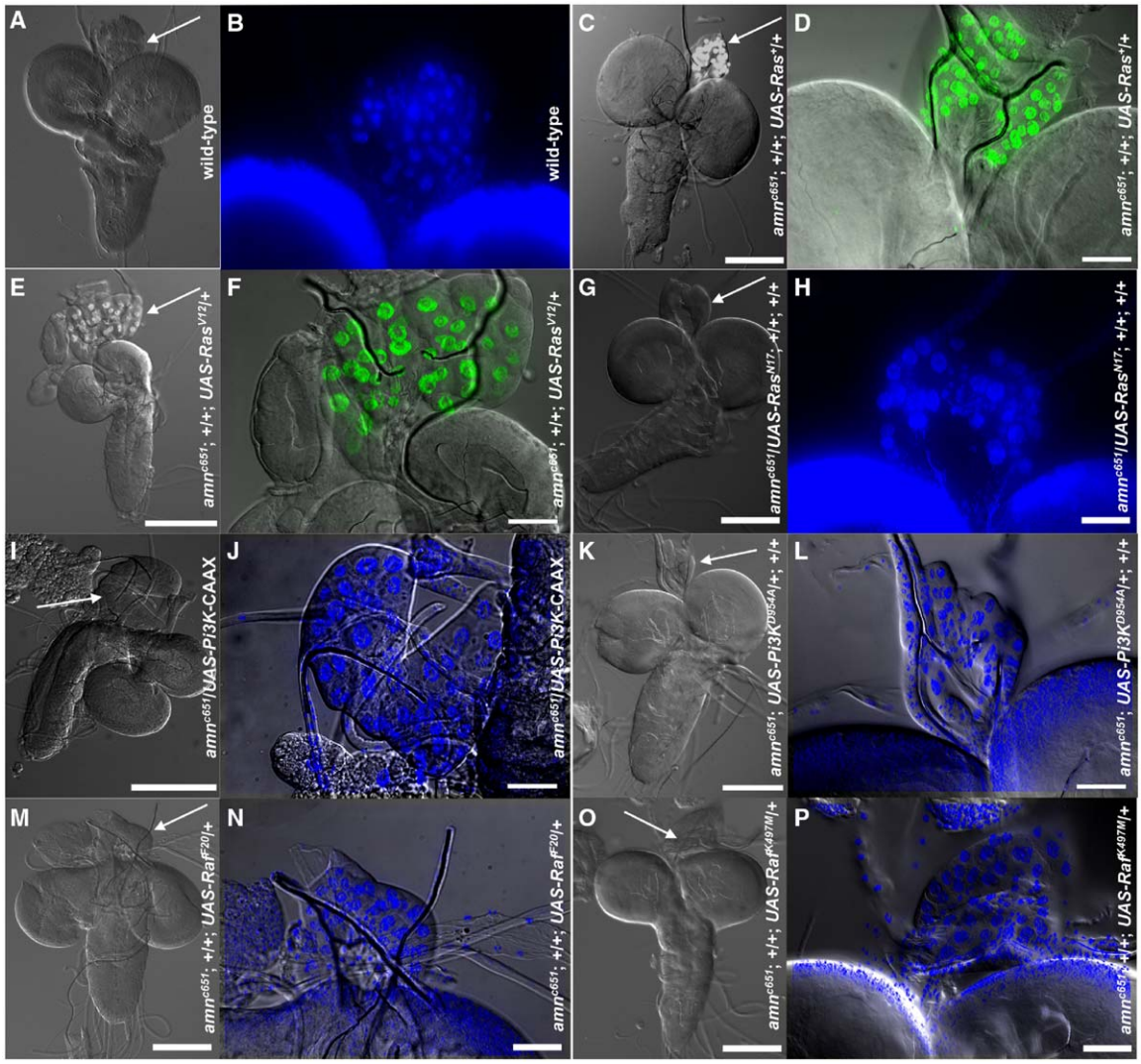

Figure 4. Altered Activity of Ras Signaling under the Control of *amn*^{c651} Affects Prothoracic-Gland Cell Size

(A and B) The prothoracic gland (PG) of a wild-type larva (*w*^{1118} and isogenic for the second and third chromosomes).
(C and D) Expression of both *Ras*$^{+}$ and nuclear-localized GFP under *amn*^{c651} control produces 50–60 normal-sized PG cells.
(E and F) Expression of both *Ras*^{V12} and nuclear-localized GFP under *amn*^{c651} control produces 50–60 enlarged PG cells.
(G and H) Expression of *Ras*^{N17} under *amn*^{c651} control has no effect on PG cell size.
(I and J) Expression of *Pi3K-CAAX* under *amn*^{c651} control increases PG cell size to the same extent as *Ras*^{V12}.
(K and L) Expression of *Pi3K*^{D954A} under *amn*^{c651} control decreases PG cell size.
(M and N) Expression of *Raf*^{F20} under *amn*^{c651} control has no effect on PG cell size.
(O and P) Expression of *Raf*^{K497M} under *amn*^{c651} control has no effect on PG cell size. Arrows point to the ring gland in (A, C, E, G, I, K, M, and O). PG cell nuclei in (B, H, J, L, N, and P) are visualized with Hoechst stain. Measure bars for (A, C, E, G, I, K, M, and O) are 200 μm, and measure bars for (B, D, F, H, J, L, N, and P) are 50 μm.

ticular, between 72 and 108 AEL in larvae in which *amn*^{c651} drove *Ras*^{V12} expression, E74B transcript levels were significantly higher than in wild-type larvae (Figure 5A). Furthermore, in larvae in which *Ras*^{V12} expression was driven by *amn*^{X8}, we observed that high levels of E74A transcripts appeared at 108 hr AEL, about 24 hr before comparable levels appeared in wild-type larvae (Figure 5B). These results indicate that *Ras*^{V12} driven by either *amn*^{c651} or *amn*^{X8} causes ecdysone levels to rise 24 hr prematurely. We suggest that this precocious ecdysone burst causes the cessation of growth and the premature pupariation that we observe.

Discussion

The insect PG releases the steroid hormone ecdysone, which triggers molting and ultimately metamorphosis in response to PTTH release from neurosecretory cells in the brain. Here, we describe the effects of manipulating Ras signaling activity in the PG of *Drosophila* larvae. We find that inhibiting Ras signaling prolongs the second- or third-instar stages (reduces the developmental rate), allowing increased growth to occur and ultimately leading to the delayed appearance of large flies. In contrast, activating Ras in the PG shortens the second- and third-instar stages (increases developmental rate),

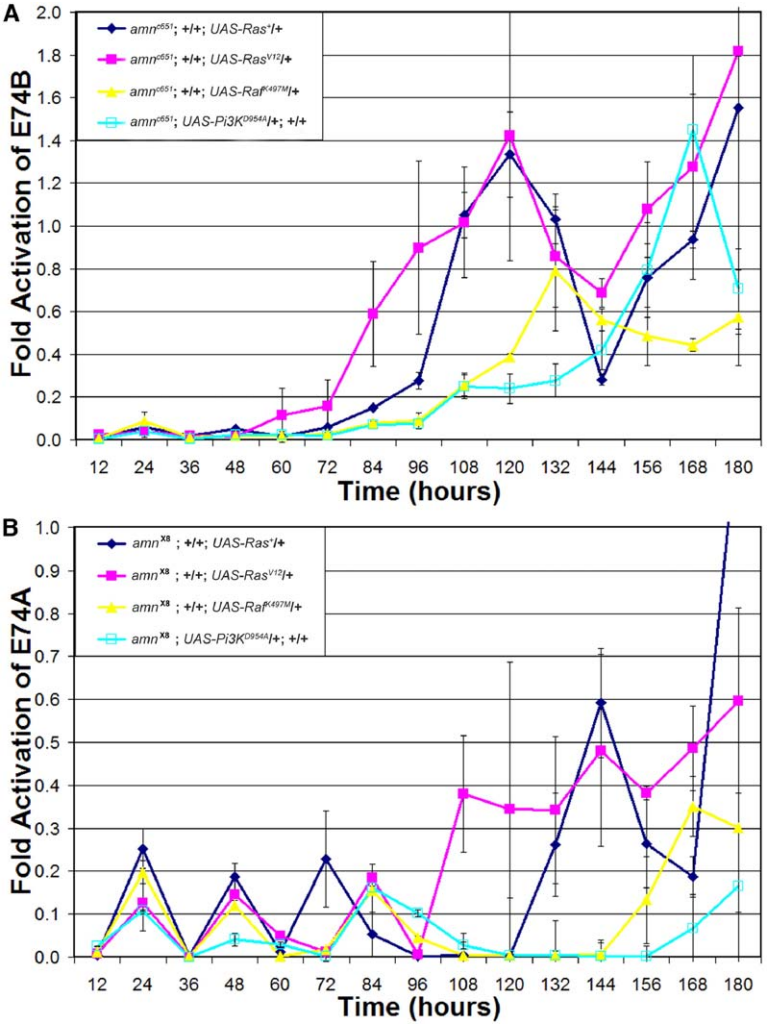

Figure 5. Altered Ras Signaling under the Control of *amn*^{c651} and *amn*^{X8} Affects the Timing of Ecdysone Release

(A) Altered Ras signaling under the control of *amn*^{c651} affects the transcriptional activation of E74B.

(B) Altered Ras signaling under the control of *amn*^{X8} affects the transcriptional activation of E74A. Mean ± SEM was determined from at least two separate samples collected from each genotype, and triplicate measures of each sample were conducted with the relative $2^{-\Delta\Delta Ct}$ method [44] to ensure consistency. Y axis: E74A or E74B transcript levels normalized to RpL13A (arbitrary units). X axis: hours AEL from which larvae were collected.

leading to premature pupariation and the generation of small pupae and adults. These effects appear to be mediated by altered ecdysone release: Inhibition of Ras signaling in the PG greatly attenuates the increase in ecdysone titer that occurs during the third-instar stage, whereas activating Ras in the PG causes a precocious increase in this titer. We conclude that altered Ras activity in the PG alters both the duration of the larval stages and final body size via the timing of ecdysone release. A model summarizing these conclusions is shown in Figure 6.

Our results suggest that both Raf and PI3K participate in the regulation of body size, developmental rate, and the timing of ecdysone release. In particular, expression of constitutively active versions of either *Raf* or *PI3K* in the PG generates small flies, whereas expression of dominant-negative (dn) versions of either *Raf* or *PI3K* attenuates ecdysone release and confers the delayed appearance of large flies. Mirth et al. (in this issue of *Current Biology* [35]) report a similar effect on developmental rate and body size in larvae expressing dn or constitutively active *PI3K* in the PG. However, Raf and PI3K mediate their size effects by distinct mechanisms. Activating PI3K, but not Raf, in the PG increases PG cell size, whereas expression of dn-PI3K, but not dn-Raf, in the PG reduces PG cell size. We suggest that the PG-growth inhibition conferred by dn-PI3K is responsible for the reduced ecdysone release that we observed; presumably, the hypertrophic PG caused by expression of activated PI3K promotes precocious ecdysone release. PG hypertrophy is also observed in mutants that exhibit reduced ecdysone release [36], raising the possibility that ecdysone inhibits PG growth via inhibition of PI3K.

Although the effect of PI3K on ecdysone release is likely due to its effect on PG size, it is less immediately clear how altered Raf activity regulates ecdysone release and body size. Ecdysone is synthesized from its precursor cholesterol by a series of dehydrogenation and hydroxylation reactions [10]. The identity of the PTTH-dependent step is not entirely clear; however, it was recently shown that at least two *Drosophila* genes that encode cytochrome P450 enzymes required for ecdysone synthesis are expressed in the PG only during or immediately prior to a molt [37]. Because PTTH secretion occurs at this time, this observation suggests that PTTH might activate ecdysone synthesis by inducing the transcription of ecdysone biosynthetic genes.

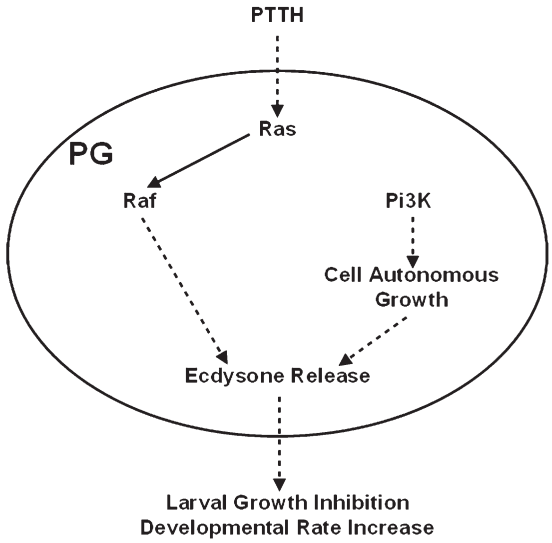

Figure 6. Proposed Model Describing the Effects of Ras Signaling on Ecdysone Release, Larval Growth Rate, and Developmental Rate

In this view, ecdysone release from the PG is regulated by two mechanisms: a PI3K-dependent cell-autonomous growth effect on PG cells, and a PTTH-induced, Ras/Raf-dependent step that may involve the transcriptional regulation of ecdysone biosynthetic genes. Ecdysone release then affects both the growth and developmental rates of larvae, resulting in final body size.

We speculate that the effects of altered Raf activity on ecdysone release might result from altered transcription of these genes.

Because PG hypertrophy is caused by either Ras^{V12} or activated PI3K but not activated Raf, we suggest that Ras^{V12} is sufficient to activate PI3K, as has been observed previously. However, the importance of Ras in the activation of PI3K under physiological conditions remains controversial [5, 38]; PI3K can be activated by several Ras-independent mechanisms, raising the possibility that activated Ras might be sufficient but not necessary for PI3K activation. Furthermore, it has been suggested that in *Drosophila*, at least, wild-type Ras normally activates PI3K poorly, and thus activation might often be an artifact of Ras^{V12} overexpression [5]. We present results that are consistent with this suggestion: Inhibiting endogenous Ras activity within the PG by expression of the dominant-negative Ras^{N17} conferred the delayed appearance of large flies but did not detectably reduce larval PG size, suggesting that PI3K is not inhibited by Ras^{N17} expression. Taken together, these observations are most consistent with the possibility that inhibiting endogenous Ras inhibits Raf, but not PI3K. In this view, PI3K in the PG is normally activated at least in part by Ras-independent factors (for example, by insulin-like peptides). Alternatively, it is possible that the inhibition of endogenous Ras by Ras^{N17} expression was not complete, and enough residual endogenous Ras remained active to permit PI3K-dependent PG growth. In either case, the observation that Ras^{N17}, like Raf^{K497M}, can slow the developmental rate and increase body size without an effect on PG size suggests that Ras is necessary for Raf activity.

A requirement for Raf supports the notion that ecdysone release is regulated differently in *Drosophila* than in *Manduca*. Although in *Manduca*, PTTH induces Erk activation [15], possibly via Raf, the role of this activation in regulating ecdysone release is unclear. Rather, a PTTH-induced increase in [cAMP] is both necessary and sufficient for ecdysone release in *Manduca* [13, 14]. In constrast, increases in [cAMP] within the PG do not directly trigger ecdysone release in *Drosophila* [16]. However, there is some evidence that altered [cAMP] might regulate ecdysone release indirectly in *Drosophila*, although these results are at present difficult to integrate with our results on regulation by Raf. First, alteration in protein kinase A (PKA) activity specifically within the PG affects the timing of molts in complex ways [39]. Second, transgene-induced alterations in PKA activity nonautonomously reduces body size in *Drosophila* [40, 41]. Third, mutations in *NF1*, the *Drosophila* ortholog of the human gene responsible for type 1 neurofibromatosis, also nonautonomously reduce fly size [25]. Although *NF1* is best characterized as a Ras GTPase-activator and thus a negative regulator of Ras, the small-size phenotype of *Drosophila NF1* mutants apparently results from reduced [cAMP] because the phenotype is completely rescued by induced expression of a constitutively active (cAMP-independent) PKA [25]. To our knowledge, it is not known whether *NF1* and PKA regulate body size via ecdysone release, but if so, a role for cAMP in regulating *Drosophila* ecdysone release would be demonstrated.

In this regard, it is noteworthy that two *Gal4* elements inserted into *amn* each express in the PG. The amn^{c651} expression pattern was suggested to mirror the endogenous *amn* expression pattern most closely [21], raising the possibility that *amn* is normally expressed in the PG. The *amn* gene is predicted to encode three neuropeptides: One is homologous to growth-hormone-releasing hormone, another is homologous to the pituitary adenylate cyclase activator peptide called PACAP, and the other is a novel neuropeptide [18]. At least one of these neuropeptides activates adenylate cyclase in *Drosophila* in vivo [19]. Additionally, *Drosophila NF1* is an essential mediator of PACAP-induced signaling at the *Drosophila* neuromuscular junction [42]. These observations raise the possibility that an Amn neuropeptide might actively participate in regulating ecdysone synthesis, rather than merely serving as a host for *Gal4* elements. However, an *amn* mutant genotype is not required for altered Ras signaling in the PG to alter body size: The *c805 Gal4* element confers Ras^{V12}-dependent reductions in body size even in an amn^{+} background (P.E.C. and M.S., unpublished data), and ectopic expression of *amn* does not rescue the body-size phenotypes conferred by amn^{c651}- or amn^{X8}-driven Ras^{V12} expression. Further investigation will be required to determine whether the Amn neuropeptides play a role in regulating ecdysone synthesis.

Conclusions

The ability of an organism to modulate body size according to environmental factors (e.g., nutrient availability) presumably confers a selective advantage, and yet the mechanisms by which these environmental fac-

tors interact with intrinsic genetic factors remain incompletely understood. One possible mechanism for this interaction comes from our observations that altered activity of Ras signaling in the *Drosophila* prothoracic gland regulates both body size and the timing of developmental events via altered release of the steroid hormone ecdysone. In mammals, growth and the timing of developmental processes (e.g., puberty) are also regulated by steroid hormones. Thus, our studies on the regulation of ecdysone release in *Drosophila* might have general biological significance.

Experimental Procedures

Drosophila Stocks

All fly stocks were maintained on standard cornmeal/agar *Drosophila* media at room temperature (22°C). The *Drosophila* Stock Center at Bloomington, Indiana provided *UAS-Ras*$^{+}$, *UAS-Ras*^{N17}, the third chromosome *UAS-Ras*^{V12}, *UAS-Raf*^{F20}, and *UAS-Raf*^{K497M}. The second chromosome *UAS-Ras*^{V12} was provided by Andreas Bergmann, *UAS-Ral*^{V20} was provided by Hideyuki Okano, and both *UAS-Pi3K-CAAX* and *UAS-Pi3K*^{D954A} were provided by Sally Leevers. The *amn*^{c651} Gal4 line was provided by Doug Armstrong, and the *amn*^{X8} Gal4 line was provided by Ulrike Heberlein.

Imaging

For fluorescent imaging, tissues were fixed in 4% paraformaldehyde dissolved in phosphate-buffered saline containing 0.2% Triton X-100 (PBST, pH 7.2). After they were rinsed in PBST, GFP containing larval tissues and all whole larvae were mounted in Vectashield medium (Vector Laboratories), and some tissues used to determine prothoracic gland cell size were mounted in Vectashield medium (Vector Laboratories) containing 0.1% Hoechst stain (Molecular Probes). All larvae and tissues were viewed on a Zeiss Axioplan 2 with Metamorph deconvolution software. For light microscopy, flies were viewed on a Leica MZFLIII dissecting microscope.

Growth Measurements

At least 10 male and female pupae and adults were measured for length for each genotype. Female wing area was determined with standard techniques [43]. Wing hair-cell density was determined by averaging the number of wing hair cells of three separate 100 μm^2 regions in the female wing between veins L3 and L4.

Fly Staging and Molting Profiles

At least 10 mating pairs were primed in a vial containing standard media for 3 days then transferred to a new vial every 12 hr. Each time point was obtained from a minimum of two separate crosses. The larvae were washed with saline solution and screened for developmental stage, via mouth hook and anterior spiracle morphology, at the appropriate time point. All staging experiments were performed at room temperature (22°C).

Quantitative PCR

Taqman primers and probes were designed with the PrimerExpress software (Applied Biosystems) according to the published sequences of E74A, E74B, and RpL13A. The E74A forward-primer sequence is (GTTGCCGGAACATTATGGATATA), the E74A reverse primer is (GCCCTATGTCGGCTTGCT), and the E74A probe is (FAM-CTTGAGATGAGGCCGCA-MGB). The E74B forward primer is (ATC GGCGGCCTACAAGAAG), the E74B reverse primer is (TCGATTGC TTGACAATAGGAATTTC), and the E74B probe is (FAM-TTGATGAA GCGATATTACAC-MGB). The RpL13A forward primer is (TCCGT GCGGTTCGTAAAAAT), the RpL13A reverse primer is (TGGCCGCG ACCATCA), and the RpL13A probe is (VIC-TGGTTTGAACAGGACC-MGB). All primers are from 5′ to 3′. Reactions for E74A, E74B, and RpL13A are of equal efficiency, as tested by standard techniques [44]. Total RNA was extracted from frozen tissues with TRIzol reagent (Invitrogen). The yield of RNA was estimated spectrophotometrically by absorbance at 260 nm (A_{260}). A_{260}:A_{280} ratio was 1.8–2:1. SuperScript II RNase H-Reverse Transcriptase (Invitrogen) treatments were carried out subsequently in accordance with the manufacturer's instructions. Reverse-transcribed cDNA from tissues was subsequently examined in a 50 μl PCR reaction by the ABI Prism 7000 system (Applied Biosystems) with the universal conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles (15 s at 95°C, 1 min at 60°C). Two separate samples were collected from each genotype, and triplicate measures of each sample were conducted to ensure the consistency of the amplification.

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